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Programmed cell death of primordial germ cells in Drosophila is regulated by p53 and the Outsiders monocarboxylate transporter

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Abstract

Primordial germ cell development uses programmed cell death to remove abnormal, misplaced or excess cells. Precise control of this process is essential to maintain the continuity and integrity of the germline, and to prevent germ cells from colonizing locations other than the gonads. Through careful analyses of primordial germ cell distribution in developing *Drosophila melanogaster* embryos, we show that normal germ cell development involves extensive programmed cell death during stages 10-12 of embryogenesis. This germ cell death is mediated by *Drosophila p53* (*p53*). Mutations in *p53* result in excess primordial germ cells that are ectopic to the gonads. Initial movements of the germ cells appear normal, and wild-type numbers of germ cells populate the gonads, indicating that *p53* is required for germ cell death, but not migration. To our knowledge, this is the first report of a loss-of-function phenotype for *Drosophila p53* in a non-sensitized background. The *p53* phenotype is remarkably similar to that of *outsiders* (*out*) mutants. Here, we show that the *out* gene encodes a putative monocarboxylate transporter. Mutations in *p53* and *out* show nonallelic noncomplementation. Interestingly, overexpression of *p53* in primordial germ cells of *out* mutant embryos partially suppresses the out germ cell death phenotype, suggesting that *p53* functions in germ cells either downstream of *out* or in a closely linked pathway. These findings inform models in which signaling between p53 and cellular metabolism are integrated to regulate programmed cell death decisions.

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Programmed cell death of primordial germ cells in *Drosophila* is regulated by p53 and the Outsiders monocarboxylate transporter

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Primordial germ cell development uses programmed cell death to remove abnormal, misplaced or excess cells. Precise control of this process is essential to maintain the continuity and integrity of the germline, and to prevent germ cells from colonizing locations other than the gonads. Through careful analyses of primordial germ cell distribution in developing *Drosophila melanogaster* embryos, we show that normal germ cell development involves extensive programmed cell death during stages 10-12 of embryogenesis. This germ cell death is mediated by *Drosophila p53* (*p53*). Mutations in *p53* result in excess primordial germ cells that are ectopic to the gonads. Initial movements of the germ cells appear normal, and wild-type numbers of germ cells populate the gonads, indicating that *p53* is required for germ cell death, but not migration. To our knowledge, this is the first report of a loss-of-function phenotype for *Drosophila p53* in a non-sensitized background. The *p53* phenotype is remarkably similar to that of *outsiders* (*out*) mutants. Here, we show that the *out* gene encodes a putative monocarboxylate transporter. Mutations in *p53* and *out* show nonallelic noncomplementation. Interestingly, overexpression of *p53* in primordial germ cells of *out* mutant embryos partially suppresses the out germ cell death phenotype, suggesting that *p53* functions in germ cells either downstream of *out* or in a closely linked pathway. These findings inform models in which signaling between p53 and cellular metabolism are integrated to regulate programmed cell death decisions.

KEY WORDS: *p53*, *outsiders*, Programmed cell death, Germ cells, Monocarboxylate transporter, *Drosophila*

INTRODUCTION

Germline precursor cells in a wide variety of animal species initially form in an extraembryonic location. To serve as the basis for the next generation, they must migrate across epithelial layers, move back into the embryo, and migrate to their target tissue, the somatic gonad precursor cells (Starz-Gaiano and Lehmann, 2001; Raz, 2004; Santos and Lehmann, 2004). Developing primordial germ cells (PGCs) share many characteristics in common with metastatic cells. These include invasive movements across epithelial cell layers, migration from their site of origin to distant target tissues, and establishing colonies at secondary locations. An essential feature of both germ cell development and metastasis is the ability to survive amidst a gauntlet of signals that would normally result in the elimination of these migrating cells through the activation of intrinsic cell death programs. The ability to thwart cell death mechanisms is a hallmark of metastatic cells and is often a major factor in tumors that are resistant to traditional cancer therapies (Jin et al., 2007; Rubinsztein et al., 2007). Many clinical pathologies result from abnormal programmed cell death (PCD). One example involving the PCD of germ cells is that over 50% of germ-line-derived tumors in children are believed to be the result of impaired PCD (Göbel et al., 2000; Schneider et al., 2001; de Silva et al., 2004; Lee, 2004; Schultz et al., 2005).

In *Drosophila melanogaster*, PGCs undergo efficient PCD during embryogenesis (Underwood et al., 1980; Technau and Campos-Ortega, 1986; Coffman et al., 2002; Coffman, 2003; Sano et al., 2005). However, the molecular machinery responsible for regulating germ cell PCD is poorly understood. Both extracellular cues and cell-autonomous determinants are thought to regulate germ cell migration and death. Maternally provided *wun2* in germ cells is necessary and sufficient for germ cell survival, and overexpression of lipid phosphate phosphatases Wun or Wun2 in somatic tissues is sufficient to trigger germ cell death (Starz-Gaiano and Lehmann, 2001; Burnett and Howard, 2003; Hanyu-Nakamura et al., 2004; Renault et al., 2004; Sano et al., 2005). The downstream effectors of Wun/Wun2 action are not known. In wild-type, *wun* or *wun2* mutant embryos, germ cell PCD does not require the function of the proapopotic genes *grim*, *reaper* or *head involution defective* (*hid*). Germ cell death is not affected by the expression of the inhibitor-of-apoptosis proteins DIAP1, DIAP2 or p35, nor is it altered by the expression of a dominant negative form of the initiator caspase Nc/Dronc (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Sano et al., 2005) (Y.Y., unpublished results). Therefore, caspase-mediated apoptosis is not the predominant mechanism of PCD in *Drosophila* germ cells.

The relatively small number of PGCs produced in the *Drosophila* embryo allows the detection of subtle differences in cell death phenotypes, providing a powerful system to study the molecular mechanisms regulating these processes. In addition, the movements of PGCs through the developing embryo are well characterized. PGCs form at the posterior pole of the embryo and divide 0-2 times to produce ~30-40 cells (Rabinowitz, 1941; Sonnenblick, 1941; Underwood et al., 1980; Technau and Campos-Ortega, 1986; Hay et al., 1988; Williamson and Lehmann, 1996). Soon after these divisions, wild-type PGCs enter mitotic arrest and remain nonproliferative until the end of embryogenesis (Sonnenblick, 1941; Deshpande et al., 1999). Another feature of wild-type PGCs is that transcription is repressed. The germ cells remain transcriptionally quiescent until stages 8-9 of embryogenesis, a point just before the germ cells begin migrating (Zalokar, 1976; Van Doren et al., 1998).

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Therefore, germ cell development requires both maternally and zygotically supplied gene products. Approximately 50% of PGCs initially formed successfully migrate and are incorporated into the gonads. Classic studies have shown that the remaining PGCs do not transdifferentiate but are eliminated (Sonnenblick, 1950; Underwood et al., 1980; Technau and Campos-Ortega, 1986).

In previous studies, we used a mutagenic screen to identify genes required for *Drosophila* germ cell development (Coffman et al., 2002). This screen isolated multiple alleles of *out*, a gene that, when mutated, disrupts germ cell death, but not migration. Embryos mutant for *out* had wild-type numbers of germ cells within the gonads plus 10-15 germ cells in ectopic locations. Ectopic germ cells were rare in wild-type embryos.

To elucidate more key components of germ cell development, we studied the literature to identify central regulators of PCD. The *p53* tumor suppressor gene was of particular interest because it had demonstrated roles in multiple forms of PCD in diverse organisms. Extensive studies have shown that p53 plays pivotal roles in genome integrity and stability (reviewed by Sutcliffe and Brehm, 2004). Mutations in *p53* are present in approximately 50% of tumors (Greenblatt et al., 1994). Remarkably, loss-of-function mutations of *Drosophila p53* alone do not result in any obvious phenotypic defects. In sensitized backgrounds, *p53* has been shown to be involved in DNA-damage-induced PCD and in growth arrest associated with tissue damage (Brodsky et al., 2000; Ollmann et al., 2000; Rong et al., 2002; Sogame et al., 2003; Brodsky et al., 2004; Jaklevic and Su, 2004; Wells et al., 2006). In situ data of *p53* transcripts reveal global maternal expression followed by zygotic expression in the PGCs and hindgut cells about half way through embryogenesis (Ollmann et al., 2000; Tomancak et al., 2002). The extensive roles of *p53* in programmed cell death and development, along with its germ cell expression pattern, prompted us to investigate potential roles for *p53* in PGC death.

Here we show that PGC elimination occurs between stages 10 and 12. Loss-of-function *p53* and *out* embryos exhibit abnormal cell death with ectopic germ cells persisting outside the gonads. Germ cell migration in *p53* mutants is normal with a wild-type number of germ cells reaching the gonads. The phenotype of *p53* mutants is remarkably similar to *out*. We have identified *out* as a gene encoding a putative monocarboxylate transporter. Genetic analyses suggest that *p53* and *out* may function in a common pathway to eliminate a subset of PGCs during embryogenesis. We discuss possible PCD mechanisms that are mediated by *p53* and its potential interactions with *out* during PGC development.

MATERIALS & METHODS

Fly stocks and breeding conditions

Flies were maintained on standard media at 25°C. The out^1, out^2, out^4 and out^5 alleles were generated in an EMS mutagenesis screen (Coffman et al., 2002). For a wild-type control, we used w^{1118}, P{w^+, *fat facets-lacZ*}, the parental strain used in the mutagenesis (Fischer-Vize et al., 1992). The KG07784 strain was generated in the Berkeley Drosophila Genome Project (Crosby et al., 2007). The *p53* alleles assayed were $p53^{5A\text{-}1\text{-}4}$, $p53^{11\text{-}1B\text{-}1}$, and $p53^{-ns}$ (Rong et al., 2002; Sogame et al., 2003). The following transgenic lines were used: P{*GAL4*::*VP16-nos*.UTR} (Van Doren et al., 1998) and UAS-*p53* (Ollmann et al., 2000).

Immunocytochemistry

Immunostaining was performed following established methods (Johansen and Johansen, 2003). Embryos were fixed in 4% paraformaldehyde. Primary antibodies used for immunostaining of embryos were: Chicken anti-Vasa (a gift from K. Howard, University College, London, UK; 1:10,000), mouse anti-β-galactosidase (40-1a Developmental Studies Hybridoma Bank; 1:50), mouse-anti-clift (Eya10H6 Developmental Studies Hybridoma Bank; 1:25) (Bonini et al., 1993). Secondary antibodies used were: biotinylated anti-mouse IgG, biotinylated anti-chicken, Alexa Fluor 488-conjugated goat anti-mouse (Invitrogen; 1:500), and Alexa Fluor 568-conjugated goat anti-chicken antibodies (Invitrogen; 1:500). The ABC Elite Kit (Vector Labs) was applied to complex the biotinylated secondary antibodies with avidin conjugated to horseradish peroxidase. Peroxidase activity was visualized using diaminobenzidine as a substrate.

Germ cell counts

Germ cells were labeled using an anti-Vasa antibody. Germ cells were counted using differential interference contrast microscopy. Staging of embryos was done based on morphological criteria (Campos-Ortega and Hartensein, 1997). For bilateral segregation assays, PGCs were scored as middle cells when the cells remained close to the midline while other PGCs had moved laterally forming bilateral clusters. For stage 14 embryos, the gonadal sheath cells were used to determine whether germ cells were inside or outside of the gonads. Our criterion for a mutant phenotype in stage 14 embryos was more than three germ cells ectopic to the gonads.

Sequencing

Genomic templates of out^1, out^2, out^4, out^5, KG07784 and wild-type (w^{1118}, P{w^+, *fat facets-lacZ*}) were PCR amplified using the TripleMaster Taq system (Eppendorf). Primers used to amplify exons 2-5 of CG8062 were: 5′-caagttggtatatgggctcacc-3′ (forward) and 5′-caagccctcgaatttctgg-3′ (reverse). The entire translated region of 3200 bp was sequenced. Sequence analyses revealed nonsense mutations in out^1, out^2 and out^5. These mutations were confirmed through repeated sequencing of both strands. No sequence changes that would affect protein coding of regions were observed in out^4 and KG07784.

Removal of lethality through chromosomal recombination

The original out^4 and out^5 X chromosomes were lethal (Coffman et al., 2002) owing to second mutations on these chromosomes. Recombination was performed to remove the lethality. Heterozygous out^4 and out^5 females were crossed to w^{1118} cv^1 wy^{74i} f^1/Y males. Recombination took place in F1 (w^{1118} cv^+ wy^+ out^- f^+ P{w^+, *fat facets-lacZ*}/w^{1118} cv^1 wy^{74i} f^1) females. These females were then crossed to FM7Z/Y males. Subsequently, viable P{w^+, *fat facets-lacZ*}/Y males were collected. Stocks were established using these recombined chromosomes and tested for retention of the *out* mutations.

Reverse transcriptase PCR

To study expression of the *out* transcript, total RNA was isolated from 0-15 hour out^1, out^2, out^4, out^5, KG07784 and wild-type embryos using Trizol (Invitrogen). RNA samples were treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contamination. First-strand cDNA synthesis was performed using AffinityScript^{TM} QPCR cDNA Synthesis Kit (Stratagene) using an oligo (dT) primer. To detect the presence of the *out* cDNA in the samples, the following primers were used for PCR: 5′-gatgccaagcaaaccacg-3′ (forward) and 5′-gcctccgtcaagataccaag-3′ (reverse) to amplify a 634 bp fragment spanning exons 3-4 of CG8062. As a positive control, constitutively expressed *ribosomal protein 49* (*rp49*)-specific primers [5′-gcgcaccaagcacttcatc-3′ (forward) and 5′-gacgcactctgttgtcgatacc-3′ (reverse)] were used to ensure the quality of the cDNA templates (O'Connell and Rosbash, 1984). To distinguish cDNA from genomic contamination, all primer pairs spanned introns. PCR was performed using Taq DNA polymerase (Eppendorf) using 35 cycles of DNA amplification.

RESULTS

Most PGC death occurs by stage 12 in wild-type embryos

To define the window of time in which germ cells are eliminated and to gain more insight into the mechanisms of germ cell death, we performed careful and extensive analyses of germ cell numbers during defined stages of development (Campos-Ortega and Hartensein, 1997). Using antibodies to Vasa as a marker for germ cells, we conducted counts of PGCs during stages 10-14 (Fig. 1 and see Table S1 in the supplementary material). During stage 10, PGCs

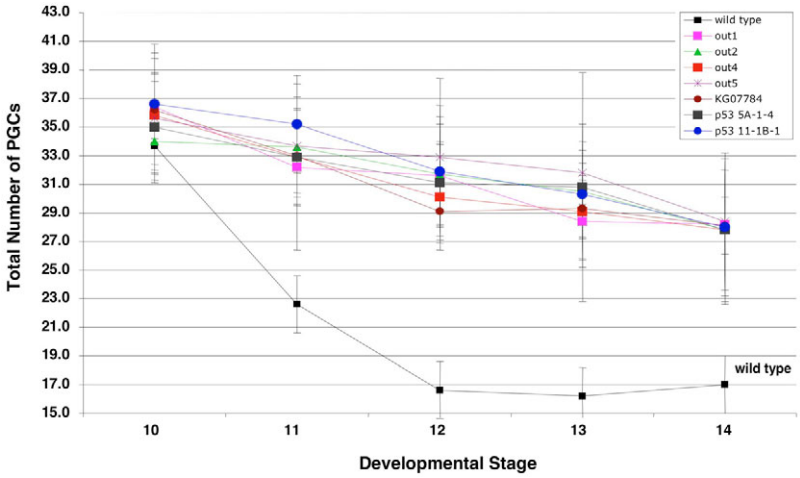

Fig. 1. Programmed cell death of migrating germ cells in wild-type, *p53* and *out* mutant embryos. Total numbers of PGCs in embryos between stages 10-14 were determined. In wild-type embryos, germ cell death was essentially complete by stage 12. In *p53* and *out* embryos, germ cell death was disrupted. Similar numbers of PGCs were observed with a gradual loss of germ cells between stages 10 and 14. Error bars represent s.d.

traversed the posterior midgut epithelium (Fig. 2A) (Warrior, 1994; Callaini et al., 1995; Jaglarz and Howard, 1995; Moore et al., 1998). They subsequently attached to overlying mesoderm. As stage 11 proceeds, the PGCs separated into two bilateral clusters and moved toward the somatic gonadal precursor cells (SGPs) (Fig. 2B). Beginning stage 12, PGCs associated with SGPs, which are specified in parasegments 10-12 (Brookman et al., 1992; Boyle and DiNardo, 1995; Boyle et al., 1997) (Fig. 2C and Fig. 3A). By stage 13, the three SGP-PGC clusters became contiguous, forming a band of cells on both sides of the embryo (Fig. 2D, Fig. 3B, Fig. 4A,B). At stage 14, PGCs and SGPs coalesced in parasegment 10 forming the early embryonic gonads (Fig. 2E and Fig. 3C).

As shown in Fig. 1 (and see Table S1 in the supplementary material), the number of Vasa-labeled germ cells in wild-type embryos dropped dramatically between stages 10 and 12. The average number of PGCs in control embryos at stage 10 was 33.7. This observation agrees with previous reports stating the numbers of germ cells present at the beginning of gastrulation, the point when the PGCs cease mitotic divisions, is in the order of 30-40 (Rabinowitz, 1941; Sonnenblick, 1941; Underwood et al., 1980; Technau and Campos-Ortega, 1986; Hay et al., 1988). Notably, the average number of PGCs at stage 11 was down to 22.6, a 33% decrease. We found that by stage 12, the average number of germ cells in the embryo was 16.6, less than 50% of the number of PGCs observed at stage 10. These observations indicate that extensive germ cell PCD took place between stages 10 and 11 as PGCs crossed the midgut epithelial layer and then transitioned into the mesoderm and moved toward the SGPs. Very little reduction in PGC numbers was observed subsequent to stage 12. We obtained similar results using a different marker for PGCs, *fat facets-lacZ* (Fischer-Vize et al., 1992) (data not shown).

p53 mutants are defective in germ cell PCD

The molecular mechanisms responsible for germ cell PCD in wild-type *Drosophila* embryos are largely unknown, but multiple observations suggested that *p53* might have a role in *Drosophila* germ cell development. First, *p53* has been shown to mediate programmed cell death in other systems (Jin, 2005; Crighton et al., 2006; Green and Chipuk, 2006). In *Drosophila*, *p53* has been shown to mediate DNA-damage-induced PCD (Jaklevic and Su, 2004; Qi

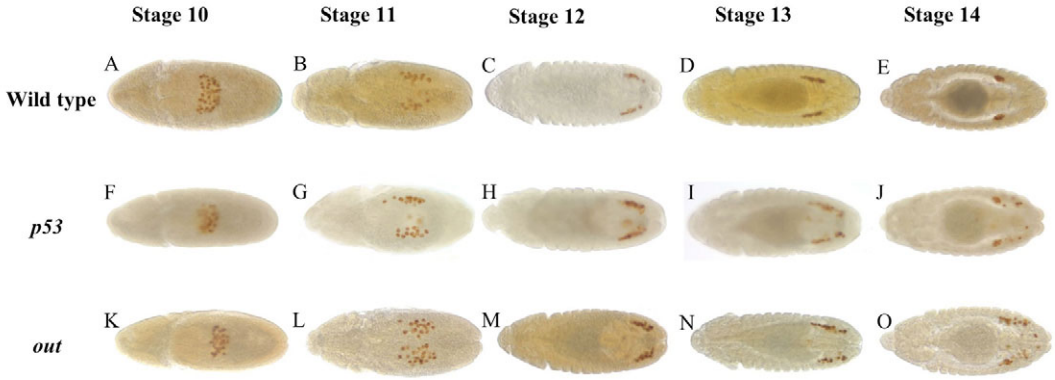

Fig. 2. Mutants initiate migration and form bilateral clusters in a similar manner to wild-type embryos. (**A-O**) Dorsal views of wild-type, *p53* and *outsiders* mutant PGC development at stages 10-14. Anterior is left in all figures. PGCs are labeled with an anti-Vasa antibody. (A) At stage 10, wild-type PGCs moved out of the posterior midgut. (F,K) The initial PGC movements in *p53* and *out* appear normal. (B) At stage 11, bilateral segregation of PGCs occurred in the wild-type embryos. (G,L) PGCs in *p53* and *out* mutants form bilateral clusters. However, there are occasionally PGCs left in the midline. (C) During stage 12, wild-type PGCs form clusters. (H,M) Bilateral clusters are also seen in *p53* and *out* mutants. Note that some PGCs remained in the midline of some *p53* embryos. (D) At stage 13, PGCs form tightly associated linear arrays of cells. (I,N) In both *p53* and *out* embryos, most PGCs were aligned. However, isolated PGCs were observed. (E) At stage 14, PGCs in wild-type embryos coalesce with SGPs. (J,O) *p53* and *out* mutant embryos exhibit very similar phenotypes. PGCs are able to migrate to the gonads; however, many PGCs persisted ectopic to the gonads.

et al., 2004). Second, *p53* RNA expression during early *Drosophila* embryogenesis coincides with the timing and location of PGC death. *p53* maternal transcripts and zygotic expression is high in mesoderm, gut (stage 10), and PGCs (stages 10-16) (Ollmann et al., 2000; Tomancak et al., 2002).

Germ cell development was examined in three recessive loss-of-function alleles of *p53*: $p53^{5A\text{-}1\text{-}4}$, $p53^{11\text{-}1B\text{-}1}$ and $p53^{-ns}$ (Rong et al., 2002; Sogame et al., 2003). In these *p53* mutants, PGCs are not appropriately eliminated during migration across the midgut epithelium and subsequent movements towards the somatic gonad precursor cells. To determine whether these extra PGCs resulted from overproduction of germ cells, PGC counts were conducted at different time points during germ cell migration (Fig. 1 and see Table S1 in the supplementary material). At stage 10, the average total number of PGCs in the wild-type embryos was 33.7. The p53 alleles averaged 35.0 ($p53^{5A\text{-}1\text{-}4}$), 36.0 ($p53^{11\text{-}1B\text{-}1}$), and 28.8 ($p53^{5A\text{-}1\text{-}4}/p53^{-ns}$) germ cells at stage 10. During stage 11, fewer than two PGCs were eliminated in *p53* mutants. This was significantly different from the wild type, where an average of 11 germ cells was eliminated ($P<0.0001$, Student's *t*-test). Over the course of PGC migration, a gradual reduction of PGCs occurred in *p53* mutants. By stage 14, the average total numbers of PGCs in the embryos were 27.8 in $p53^{5A\text{-}1\text{-}4}$, 28.0 in $p53^{11\text{-}1B\text{-}1}$ and 26.4 in $p53^{5A\text{-}1\text{-}4}/p53^{-ns}$. The wild-type average was 17.0. Therefore, the initial numbers of germ cells was the same in wild-type and *p53* mutants, and since the total number of germ cells slowly decreased over time, it seems unlikely that the additional PGCs observed were due to premature resumption of mitoses.

Next, we examined the requirements for maternal and zygotic expression of *p53*. Penetrance of the mutant phenotype in homozygous mutant stocks was 93% and 96% for the $p53^{5A\text{-}1\text{-}4}$ and $p53^{11\text{-}1B\text{-}1}$ alleles, respectively (Table 1). When *p53* homozygous mutant mothers were crossed to wild-type males, 30-35% of these heterozygous (*p53*/+) embryos displayed abnormal germ cell death. When heterozygous *p53*/+ mothers were crossed to homozygous mutant *p53*/*p53* males, half of the embryos were homozygous for the mutant *p53*. The penetrance of the mutant phenotype in these embryos was 42%. Thus although there was a maternal effect for *p53*, the role of zygotic expression of the gene accounted for most of the PGC phenotype.

Initial phases of PGC migration are not disrupted in *p53* mutants

We observed PGCs ectopic to the gonads at stage 14 (Fig. 2 compare E with J, and Fig. 3 compare C with F). We considered several models to explain the presence of these PGCs ectopic to the gonads of *p53* mutants. First, it could represent a defect in the ability of germ cells to cross the posterior midgut epithelium and transition into the surrounding mesoderm as in *tre1* and *slam* mutants (Kunwar et al., 2003; Stein et al., 2002). Second, *p53* mutant germ cells might fail to respond to midline repulsive signals such as those mediated by *wun* and *wun2* (Sano et al., 2005). Third, the PGCs in *p53* mutants might not successfully coalesce with somatic gonad precursor cells. Fourth, the *p53*-defective PGCs may not respond to death signals that eliminate errant germ cells in wild-type animals.

Because differences in germ cell PCD were noted at stage 11, we inspected where PGCs are positioned at this stage. A failure of germ cells to exit the posterior midgut and transition to the mesoderm results in a phenotype where the germ cells remain associated with the endoderm. Analyses of PGC locations during stages 11 and 12 showed that *p53* mutant germ cells exited the PMG (Fig. 2G,H and Fig. 3D).

Table 1. *p53* mutant embryos are defective in germ cell PCD

Female	Male	*n*	% mutant
$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	166	93
$p53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	$p53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	158	96
$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	$p53^{-ns}/p53^{-ns}$	109	75
$p53^{-ns}/p53^{-ns}$	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	55	69
$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	+/+	91	30
$p53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	+/+	204	35
$+/p53^{5A\text{-}1\text{-}4}$	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	165	42
$+/p53^{11\text{-}1B\text{-}1}$	$p53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	216	42
out^1/out^1	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	135	77
out^2/out^2	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	445	84
+/+	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	174	3
out^1/FM7Z; $p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	FM7Z/Y; $p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	95	96

Subsequent to exiting the PMG the germ cells separate into two clusters of cells. It had been shown that the functions of *wunen*/*wunen2* (*wun*/*wun2*) expressed in the central nervous system were necessary and sufficient to direct bilateral segregation of PGCs during stage 11 (Sano et al., 2005). We investigated whether the PGCs in *p53* mutants, including those fated to become ectopic PGCs, were able to respond to *wun*/*wun2* guidance cues and form bilateral clusters. To address this issue, we counted PGCs that failed to respond to midline repulsive signaling and remained in the middle of the embryo (see Table S2 in the supplementary material). We found averages of 1.9 PGCs in $p53^{5A\text{-}1\text{-}4}$, 2.5 PGCs in $p53^{11\text{-}1B\text{-}1}$ and 0.3 PGCs in $p53^{5A\text{-}1\text{-}4}/p53^{-ns}$ left in the midline of stage 11 embryos. These numbers were similar to the wild-type average of 1.8. Thus, PGCs in *p53* mutants successfully migrated away from the midline. Counts of PGCs at the midline in stage 12 and 13 *p53* embryos showed that PGCs continued to stay organized in two elongated bilateral clusters, largely avoiding the midline. Notably, between stages 11 and 13, the midline PGCs in wild-type embryos appeared to undergo PCD, the average number of midline PGCs declined from 1.8 to 0.4 between stages 11 and 12. This reduction in PGC number at the midline did not occur in *p53* mutants.

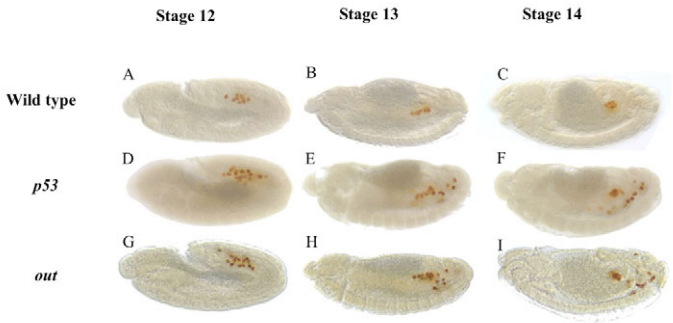

Fig. 3. Germ cells initiate migration, but those ectopic to the gonads fail to die. (**A-I**) Lateral views of wild-type, *p53* and *outsiders* mutant PGC development at stages 12-14. Anterior is left and dorsal up in all figures. PGCs are labeled with an anti-Vasa antibody. (A,D,G) PGCs in stage 12 embryos migrate into the mesoderm. (B) At stage 13, PGCs form a band of cells and are associated with one another in wild-type embryos. (E,H) In *p53* and *out* embryos, some PGCs form clusters, but isolation of several PGCs becomes apparent. (C) At stage 14, wild-type PGCs reach the gonads. (F,I) In the mutants, PGCs successfully migrated to the gonads whereas errant PGCs are found ectopic to the gonads.

To investigate whether *p53* mutant PGCs could correctly migrate towards and associate with SGPs, we double labeled embryos with a SGP marker (anti-EYA-antibody) (Boyle et al., 1997) and a PGC marker (anti-VASA-antibody). SGPs are specified at stage 11 in bilateral clusters of 9-12 cells in parasegments 10 to 12. We found that PGCs in stage 11 *p53* mutants were able to properly form clusters moving towards the SGPs (Fig. 4E,F). Also, at stage 13, double labeling for PGCs and SGPs showed alignment of PGCs with SGPs (Fig. 4G,H). However, isolation of some PGCs was also noted (Fig. 4G arrows).

PGC incorporation into the gonads was similar in wild-type and *p53* mutants (Fig. 5 and see Table S3 in the supplementary material). PGC counts of stage 14 embryos showed wild-type numbers of germ cells were successfully incorporated into the gonads in *p53* mutants. The average numbers of intragonadal germ cells were 18.9 in *p53* mutants. In the wild type, the average was 16.5. The average numbers of germ cells ectopic to the gonads were 9.1 ($p53^{5A\text{-}1\text{-}4}$), 9.4 ($p53^{11\text{-}1B\text{-}1}$) and 6.5 ($p53^{5A\text{-}1\text{-}4}/p53^{-ns}$), whereas the average in wild-type embryos was 0.4.

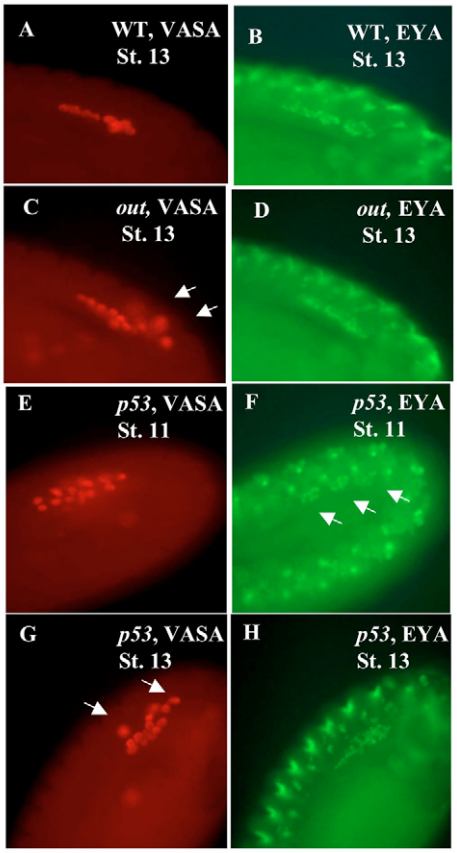

Fig. 4. SGPs form normally in mutants, but some germ cells fail to associate with SGPs. (**A**) Stage 13 wild-type embryo. PGCs form elongated clusters. (**B**) Stage 13 wild-type embryo. SGPs align with the PGCs. (**C**) Stage 13 *out* embryo. Arrows indicate errant germ cells that failed to align with SGPs. (**D**) Stage 13 *out* embryo. SGP formation appears normal. (**E**) Stage 11 *p53* embryo. PGCs migrated toward the SGPs. (**F**) Stage 11 *p53* embryo. SGPs were specified in three clusters in parasegments 10-12 (arrows). (**G**) Stage 13 *p53* embryo. Arrows indicate errant germ cells that failed to align with SGPs. (**H**) Stage 13 *p53* embryo. SGPs appear normal. Anterior is left in A-F, and downwards in G and H. A, C, E and G are stained with a Vasa antibody, and B, D, F, and H are stained with an EYA antibody.

Collectively, these data support the conclusion that mutations in *p53* result in survival of additional germ cells ectopic to the gonads because of impaired PCD rather than delays in the initiation of migration, an inability of the germ cells to leave the midline of the embryo or because of a failure of normal numbers of germ cells to associate with SGPs.

out germ phenotypes are strikingly similar to *p53*

In previous studies, we isolated six alleles of the *out* gene (Coffman et al., 2002). These preliminary studies indicated that the programmed cell death of *out* germ cells was disrupted. The similarities between the germ cell phenotypes of *out* and *p53* prompted us to investigate the out phenotype in greater depth. We examined germ cell development patterns in five *out* alleles (out^1, out^2, out^4, out^5 and KG07784), four from our original screen plus an amorphic P-element-containing line (see below) (Crosby et al., 2007), to look for similarities and differences between *out* and *p53* mutants.

As shown in Fig. 1 and see Table S1 in the supplementary material, the total numbers of germ cells observed in *out* and *p53* mutants overlapped, and PGC elimination followed a parallel pattern during stages 10 to 14. As shown in Figs 2 and 3, the general features of germ cell development were the same in *out* and *p53* embryos. Double labeling of PGCs and SGPs (Fig. 4A-D) showed that in *out* mutants, the somatic gonad cells formed normally, and germ cells were able to coalesce with SGPs. However, there were subtle differences. All of the *out* alleles had slightly more germ cells ectopic to the gonads at stage 14 than the *p53* alleles (Fig. 5 and see Table S3 in the supplementary material). This may reflect differences in genetic background because we observed strain-specific differences in germ cell numbers. Interestingly, the out^1, out^4 and out^5 alleles may represent an allelic series that reflects the severity of the amino acid truncation of the protein (see below) with out^2 being the most severe. This was apparent when the number of germ cells ectopic to the gonads (Fig. 5 and see Table S3 in the supplementary material) and the number of germ cells that failed to migrate away from the midline (see Table S2 in the supplementary material) were compared.

p53 and *out* interact genetically and overexpression of *p53* suppresses the out phenotype

The similarities between the *p53* and *out* phenotypes were intriguing. This prompted us to test the hypothesis that *p53* and *out* were involved in common PCD signaling networks. To do this, we tested for genetic interactions by creating embryos that were mutant for both *p53* and *out*.

First, we asked whether *p53* and *out* were required for the death of the same or different subsets of germ cells. If *p53* and *out* function in a common PCD signaling pathway, the number of germ cells that fail to die in double mutants would be very similar to that of either *p53* or *out* single mutants. Alternatively, if *p53* and *out* function in separate pathways responsible for elimination of different germ cells, the number of surviving germ cells ectopic to the gonads would be greater in double mutant embryos. In order to address this possibility, out^1; $p53^{5A\text{-}1\text{-}4}$ double mutants were assayed (Fig. 6E and see Table S3 in the supplementary material). Germ cell counts showed that the number of germ cells ectopic to the gonads was only slightly lower in out^1; *p53* double mutants (12.0) compared with out^1 mutants (13.4). Wild-type numbers of germ cells were incorporated into the gonads: 13.2 and 14.8 in the double mutants and out^1

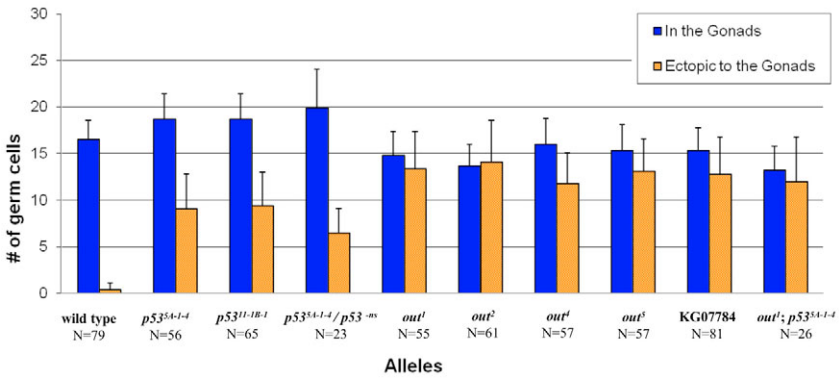

Fig. 5. Germ cells ectopic to the gonads persist in *p53* and *out* mutants. For each embryo examined, PGCs in the gonads (blue bars) and PGCs ectopic to the gonads (orange bars) were determined. PGC incorporation into the gonads was similar in wild-type, *p53* and *out* embryos. In both *p53* and *out* mutants, many ectopic PGCs were observed. In wild-type embryos, an average of 0.4 PGCs were found ectopic to the gonads. Error bars represent s.d.

mutants, respectively. This evidence suggested that *p53* and *out* functions are required for the elimination of the same subset of germ cells.

When two genes regulate common signaling pathways, transheterozygous combinations of the mutant alleles will sometimes show nonallelic noncomplementation. We reasoned that if *p53* and *out* were part of the same PCD signaling network, *p53* mutations might fail to complement *out* mutations. To test this, we produced transheterozygous embryos. Interestingly, when *out*/*out*; +/+ females were crossed to +/Y; *p53*/*p53* males, the offspring (*out*/+; *p53*/+ and *out*/Y; *p53*/+) were 77% and 84% mutant for out^1 and out^2 alleles, respectively (Table 1). When out^1/out^1 or out^2/out^2 females were crossed to wild-type males, 66% and 67% (out^1 and out^2, respectively) of the offspring displayed abnormal germ cell PCD (see Table S4 in the supplementary material). When wild-type females were crossed to +/Y; $p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$ males, only 3% of the offspring were scored as having more than three germ cells ectopic to the gonads. Therefore, the transheterozygous combination of *out* and *p53* increased the penetrance of the PCD phenotype observed.

Finally, we examined the hypothesis that *p53* functions downstream of *out* in signaling the death of germ cells that are ectopic to the gonads. To do this, we overexpressed *p53* specifically in PGCs in *out* mutant embryos using the *nos-Gal4:VP16* germ-cell-specific driver (Van Doren et al., 1998). The results showed that *p53* expression in PGCs can partially rescue the defective germ cell PCD in *out* mutants (Table 2). When there was no *p53* expression in PGCs, 47% of the embryos displayed the mutant phenotype. When *p53* expression was driven in PGCs, 32% of the embryos had more than three germ cells ectopic to the gonads. *p53* expression in wild-type embryos did not affect survival of PGCs in the gonads. These data support a model where *p53* functions downstream of, or in parallel to *out* to induce PCD when expressed in PGCs.

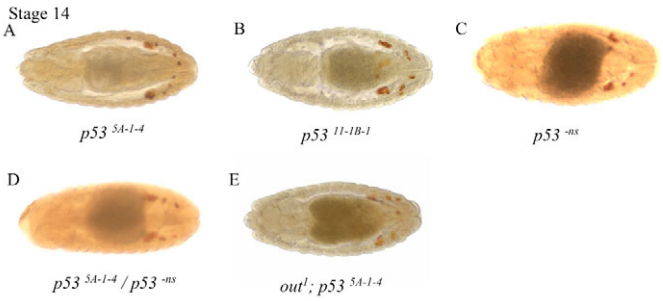

Fig. 6. The *p53* germ cell phenotype is similar to the *out*; *p53* double mutant. Dorsal views of the *p53* mutants at stage 14 are shown. (**A**,**B**,**C**) *p53* mutants displayed germ cells ectopic to the gonads. (**D**) A transheterozygous $p53^{5A\text{-}1\text{-}4}/p53^{-ns}$ mutant embryo is shown. (**E**) The out^1;*p53* double mutant appears very similar to the *p53* mutants. The germ cells in the double mutant are labeled with an anti-β-gal antibody.

Mapping of *out*

To gain further understanding of *out*- and *p53*-mediated germ cell PCD, we determined the molecular identity of *out*. In previous studies we had determined that the *out* mutant germ cell phenotype was uncovered by a deletion Df (1)JA27, which removed the 18A-18D region of X-chromosome (Coffman et al., 2002). To more narrowly define the region, we performed recombination mapping, resolving the location of *out* the 18B-C interval. With this information, we tested for non-complementation of *out* with P-element lines containing inserts in 18B-C (Crosby et al., 2007). Among the P-element lines tested, KG07784, a P-element insertion in 18C, failed to complement *out* (see Table S4 in the supplementary material). Test crosses showed that KG07784 was recessive to the wild type, and crosses between KG07784 and the *out* alleles resulted in over 90% mutant embryos.

The KG07784 P-element was reported to be inserted into the first intron of the gene CG8062 (Crosby et al., 2007). We confirmed the location of the P-element insertion by performing inverse PCR. Recovered flanking sequences were located in the first intron of CG8062 (Fig. 7A). These results provided preliminary evidence that disruption of CG8062 caused defective PCD of the ectopic germ cells.

Predicted molecular function of the Out protein

The CG8062 sequence is predicted to encode a protein of 655 amino acids encoding a potential monocarboxylate transporter (MCT) (Fig. 7B). Secondary structure prediction and domain analysis programs indicated 12 potential transmembrane domains,

Table 2. Partial rescue of *out* mutant phenotype through PGC expression of *p53*

Female	Male	*n*	% mutant
+/+; *nos*-GAL4/*nos*-GAL4*	W^{1118}/Y	219	8
+/+; *nos*-GAL4/*nos*-GAL4	UAS-*p53*/UAS-*p53*	69	3
out^2/out^2; *nos*-GAL4/*nos*-GAL4	W^{1118}/Y	133	47†
out^2/out^2; *nos*-GAL4/*nos*-GAL4	UAS-*p53*/UAS-*p53*	90	32†

*nos-GAL4:VP16.
†*out* is X-linked: 50% of offspring are out/+ and 50% are *out*/Y.

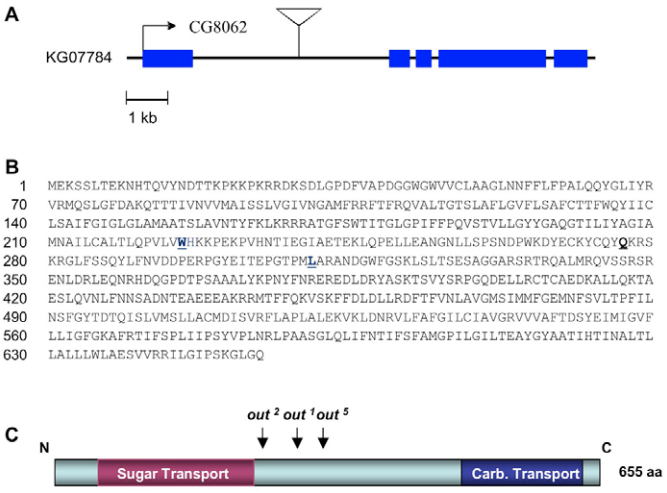

Fig. 7. Molecular identification of *out*. (**A**) A P-element insertion in CG8062 disrupts germ cell PCD. This allele fails to complement other *out* mutants. The P-element lies between exons 1 and 2. (**B**) Amino acid sequence of OUT. Three *out* alleles introduce premature stop codons in the CG8062 transcript. The wild-type OUT peptide comprises 655 amino acids. The positions of the stop codons are indicated by the bold underlined letters. (**C**) Schematic of the predicted OUT protein. Positions of the *out* nonsense mutations are indicated by arrows. The introduced stop codons would result in truncated proteins of 224, 276 and 310 amino acids for out^2, out^1 and out^5, respectively.

a sugar transporter domain, and a carbohydrate transporter domain (Fig. 7C) (Krogh et al., 2001; Juretic et al., 2002; Marchler-Bauer and Bryant, 2004). The *Drosophila* genome contains 18 genes predicted to encode MCTs (Crosby et al., 2007), but little is known about their molecular or developmental functions. Studies in other organisms have shown that MCTs localize in the plasma membrane and/or mitochondrial membrane, allowing trafficking of molecules such as lactate, pyruvate and protons, which are all major factors in cell metabolism (Halestrap and Price, 1999; Enerson and Drewes, 2003; Izumi et al., 2003; Halestrap and Meredith, 2004; Pierre and Pellerin, 2005).

Molecular characterization of *out* alleles reveals premature stop codons in out^1, out^2 and out^5

We predicted that significant *out* mutations should be present in CG8062 to give such a severe germ cell PCD defect. out^1, out^2, out^4, out^5, KG07784 and wild-type genomic templates were PCR amplified and sequenced. The amplified 3200 bp included exons 2-5, the translated regions of the gene. We found nonsense mutations in out^1, out^2, and out^5 (Fig. 7B,C). The predicted proteins encoded by the *out* mutants would be 224 amino acids in out^2, 276 amino acids in out^1, and 310 amino acids in out^5. In out^4, a single basepair change from G to A at a splice donor site preceding the conserved GT-intron border is predicted to significantly reduce its likelihood of being a splicing junction (Crosby et al., 2007). No significant basepair changes were found in exons 2-5 of KG07784. The P-element insertion in the intronic region of CG8062 may interfere with transcription or processing of the transcript. To detect the presence of the CG8062 transcript in KG07784, we used reverse-transcriptase PCR. The CG8062 transcript was detected in out^1, out^2, out^5 and the wild type, but was absent in KG07784 mutants (see Fig. S1A in the supplementary material). As a control, we assayed for the presence of a transcript from the *ribosomal protein 49* gene (*rp49*) to ensure the quality of the cDNA. The *rp49* transcript was detected in KG07784. We concluded that KG07784 is a transcript null allele of CG8062.

DISCUSSION

Drosophila PGC death is mediated by *p53* and *out*

We provide evidence that *p53* and *out* are required for the elimination of excess PGCs in the early stages of *Drosophila* development. Loss-of-function alleles of both genes result in PGCs that persist in ectopic locations, whereas wild-type numbers of PGCs are incorporated into the gonads. Germ cell movements are not delayed, and the PGCs appear to respond to midline repulsive signals, separating into two bilateral clusters. We conclude that *p53* and *out* are necessary for PCD, but not for migration. Interestingly, *p53* and *out* appear to mediate common PCD signaling networks to eliminate the same populations of ectopic germ cells. Although the molecular mechanisms are not known, our observation that overexpression of *p53* in germ cells is able to suppress the effects of mutations in *out* suggests that *p53* may be acting downstream of *out*. To our knowledge, this is the first report of a phenotype associated with loss-of-function alleles of *Drosophila p53* in a nonsensitized background.

Roles for *p53* in germ cell development and PCD

Studies in other metazoans have shown significant roles of *p53* in germline development. In *C. elegans*, the *p53* homolog *cep-1* is required for proper chromosome segregation during meiosis and DNA-damage-induced germ cell death (Derry et al., 2001). In mice, *p53* acts to maintain the integrity of the germ line. Mice lacking *p53* exhibited reduced spontaneous germ cell death and increased levels of abnormal sperm (Beumer et al., 1998; Yin et al., 1998). Furthermore, murine *p53* has been shown to positively regulate PGC apoptosis associated with loss of Connexin 43, a gap junction component expressed in PGCs (Francis and Lo, 2006). PGCs in Connexin-43-knockout mice exhibit abnormally increased levels of activated *p53* and apoptosis. This increased PGC death can be rescued by injections with a p53 inhibitor. These observations, together with our work, clearly show that *p53* is an essential mediator of germ cell PCD.

Our observations indicate that PGCs in wild-type embryos undergo extensive PCD between stages 10-12 of embryogenesis. However, the mechanisms of *Drosophila* PGC death are not understood. Accumulating evidence suggests that *Drosophila* germ cell PCD is context dependent, and both apoptotic and non-apoptotic cell death can occur. Embryos homozygous for the Df (3L)H99 deletion, which removes the potent apoptotic inducers *grim*, *rpr* and *hid*, show normal germ cell PCD (Sano et al., 2005) (Y.Y., unpublished results). Altered expression of the caspase inhibitors p35, DIAP1, DIAP2 or a dominant negative form of Nc/Dronc did not affect Wun/Wun2-mediated PGC death (Hanyu-Nakamura et al., 2004; Renault et al., 2004). Cells dying in response to Wun/Wun2-mediated signals were negative for TUNEL staining and did not label for another marker of apoptosis, cleaved caspase 3. These observations argue that during normal development, PGC death does not occur by apoptosis. However, it is important to note that germ cells are capable of undergoing caspase-mediated PCD. Expression of *hid* or *rpr* in PGCs induces extensive PCD (Sano et al., 2005) (Y.Y., unpublished). In addition, germ cells mutant for *nanos* fail to maintain the germ cell fate and undergo apoptosis (Hayashi et al., 2004).

There are reports demonstrating that *p53* has roles in caspase-independent modes of PCD (Feng et al., 2005; Coureuil et al., 2006; Crighton et al., 2006). For example, death of terminally differentiating murine germ cells induced by *p53* overexpression is mediated by calpains rather than caspases (Coureuil et al., 2006).

There is accumulating evidence that PCD is context dependent and that cell death does not always occur by a single mechanism (Edinger and Thompson, 2004; Lockshin and Zakeri, 2004). Therefore, the possibility of crosstalk between different cell death pathways and hybrid forms of cell death need to be considered. For example, when apoptosis is blocked by caspase inhibition, mammalian neurons can still undergo PCD via autophagy (Lang-Rollin et al., 2003). This raises an intriguing possibility that PGCs use multiple cell death mechanisms. In support of this hypothesis, ectopic PGCs, which normally undergo Bax-mediated apoptosis, still undergo PCD in Bax-deficient mouse embryos suggesting that there must be Bax-independent PCD mechanisms (Stallock et al., 2003).

p53 expression in PGCs eliminates errant PGCs in *out* mutants

Our data show that *p53* genetically interacts with *out*, a predicted MCT, in germ cell PCD. Additionally, *out* and *p53* show nonallelic noncomplementation suggesting that they may be involved in closely linked functions. Partial rescue of the defective germ cell PCD by overexpression of *p53* in *out* mutants suggests that *p53* may function downstream of *out*. This incomplete zygotic rescue may reflect the fact that *p53* shows a maternal effect. Alternatively, *p53* and *out* may function in parallel pathways. Importantly, PGC expression of *p53* in wild-type embryos does not affect PGCs within the gonads. Forced expression of *p53* induces PCD only in the errant PGCs in *out* mutants. These observations implicate potential mechanisms that distinguish between subpopulations of germ cells. Such mechanisms may involve activation of downstream antagonists of *p53*-mediated PCD in surviving PGCs.

The MCT family, of which *out* is a member, consists of eighteen predicted *Drosophila* genes (Crosby et al., 2007). Very little is known about their cellular functions. The mammalian MCT family of proteins includes 14 members, 4 of them with experimentally demonstrated functions for catalysis of the proton-linked transport of monocarboxylates (Halestrap and Meredith, 2004). It has been shown that MCTs localize at the plasma membrane and/or mitochondrial membranes, and their substrates include major factors in cellular metabolism such as lactate and pyruvate (Halestrap and Price, 1999).

Some recent reports suggest interesting possibilities for links between MCTs, cell metabolism, p53 and programmed cell death (Bensaad and Vousden, 2007; Danial et al., 2003; Feng et al., 2007). First, low nutrient levels negatively regulate mTOR and promote autophagy (Kamada et al., 2004; Lum et al., 2005). mTOR, together with insulin-like growth factor 1, monitors levels of nutrients and mitogens to regulate cell growth and division. Downstream components of these regulators include Akt-1 kinase. Akt negatively regulates both autophagy and apoptosis (Rasoulpour et al., 2006; Quevedo et al., 2007). *Caenorhabditis elegans* homologs of Akt have been shown to suppress DNA-damage-induced germ cell death, involving *cep-1*, the *p53* homolog (Quevedo et al., 2007). Germ cell death was significantly decreased in *akt-1* gain-of-function mutants. Loss of *cep-1*/*p53* completely blocks apoptotic hypersensitivity in *akt-1* loss-of-function mutants.

Second, the functions of the SLC5A8 Na^+-coupled MCT were linked to induction of pyruvate-dependent inhibition of histone deacetylases (HDAC), elevated levels of *p53* and apoptosis in tumor cell cultures, suggesting pro-PCD functions of this MCT within the dying cells (Thangaraju et al., 2006). Although the exact mechanism as to how pyruvate uptake leads to cell death is unknown, the correlation with upregulated *p53* expression and HDAC inhibition is intriguing. Interestingly, studies in *Drosophila* wing development show that cell death mechanisms require functions of a histone acetyltransferase (HAT). This activity is antagonized by a HDAC (Miotto et al., 2006). Additionally, *p53* function is linked to histone acetylation. It has been shown that *p53* is important for maintenance of histone H3 acetylation after irradiation (Rebollar et al., 2006).

Drosophila PGCs appear capable of undergoing multiple forms of PCD. Our demonstration that *p53* is involved in some form of PGC death suggests interesting hypotheses to test. Our observations of pro-cell death roles for *out*, an MCT that can be suppressed by *p53* overexpression, provide tantalizing clues. Clearly *p53* and *out* are two pieces of a much larger puzzle potentially linking cell metabolism to cell death or survival signaling.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/2/207/DC1

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